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ORIGINAL ARTICLE

# Cyclosporine A from a nonpathogenic *Fusarium oxysporum* suppressing *Sclerotinia sclerotiorum*

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## Abstract

**Aims:** To evaluate the antagonistic activity of *Fusarium oxysporum* nonpathogenic fungal strain S6 against the phytopathogenic fungus *Sclerotinia sclerotiorum* and to identify the antifungal compounds involved.

**Methods and Results:** The antagonistic activity of *Fusarium oxysporum* strain S6 was determined *in vitro* by dual cultures. The metabolite responsible for the activity was isolated by chromatographic techniques, purified and identified by spectroscopic methods as cyclosporine A. The antifungal activity against the pathogen was correlated with the presence of this metabolite by a dilution assay and then quantified. Cyclosporine A caused both growth inhibition and suppression of sclerotia formation. In a greenhouse assay, a significant increase in the number of surviving soybean (*Glycine max*) plants was observed when *S. sclerotiorum* and *F. oxysporum* (S6) were inoculated together when compared with plants inoculated with *S. sclerotiorum* alone.

**Conclusion:** *Fusarium oxysporum* (S6) may be a good fungal biological control agent for *S. sclerotiorum* and cyclosporine A is the responsible metabolite involved in its antagonistic activity *in vitro*.

**Significance and Impact of the Study:** Cyclosporine A has not been previously described as an inhibitor of *S. sclerotiorum*. Its minimum inhibitory concentration (MIC) of 0·1 $\mu$g disc$^{-1}$ makes it suitable to use as a biofungicide. *In vivo* experiments showed that *F. oxysporum* (S6) is a good candidate for the biocontrol of *S. sclerotiorum* in soybean.

## Introduction

*Sclerotinia sclerotiorum* is a soil-borne pathogen with a wide host range including many of economical importance (Boland and Hall 1994). This pathogenic fungus produces significant losses in soybean (*Glycine max*), sunflower (*Helianthus annuus*) and lettuce (*Lactuca sativa*) crops (Zhou and Boland 1998). *Sclerotinia* species produce mycelia and sclerotia (anamorph) and apothecia, asci and ascospores (teleomorph) (Kohn 1979). Sclerotia are viable for more than 5 years (Le Tourneau 1979) and may germinate by producing either mycelia (myceliogenic germination) or apothecia (carpogenic germination). During myceliogenic germination, the hyphae grow towards host roots and hypocotyls causing shoot wilt (Adams and Ayers 1979).

Several methods of disease control such as agrochemicals, use of resistant cultivars and cultural practices have been used. However, none of them has been completely successful (Adams and Ayers 1979; Whipps and Budge 1990). Biocontrol may be an environmentally friendly and efficient alternative to manage pathogenic fungi (Huang 1992; Whipps 2001).

Some soils, called suppressive soils, inhibit fungal mycelia and/or spore germination, a phenomenon known as soil fungistasis. In fact, there are several diseases in which the pathogen cannot develop naturally because of the soil. Previous reports have determined that micro-organisms have an important role as causal agents of fungistasis, with their action mediated either by available carbon limitation or by production of

 

antifungal compounds (de Boer *et al.* 2003). For this reason, suppressive soils are an important source of biocontrol agents. Several studies have used this natural advantage to develop effective biological control strategies (Butt *et al.* 2001; Whipps 2001).

*Fusarium oxysporum* is one of the most distributed species in soil-borne fungi communities, particularly in plant rhizospheres (Gordon and Martyn 1997), where pathogenic and nonpathogenic strains may be found. Some strains of *F. oxysporum* have shown the ability to suppress the growth of several fungal plant pathogens such as *Phytophtothora erythroseptica* and *Pythium ultimum* (Park 1963; Benhamou *et al.* 2002) and to affect the germination of *S. sclerotiorum* sclerotia (Zazzerini and Tosi 1985). Other species of *Fusarium* have been evaluated both against *P. ultimum* (Ishimoto *et al.* 2004) and as a potential biocontrol agent against *S. sclerotiorum* in the rhizosphere (Zhou and Boland 1998). Little is known about the antagonism related with antifungal metabolite production by nonpathogenic *F. oxysporum* (Fravel *et al.* 2003).

As part of our continued interest in the studies on biocontrol fungal agents against the fungal plant pathogen *S. sclerotiorum*, suppressive soils from fields cropped with soybean were investigated. A nonpathogenic strain of *F. oxysporum* with antagonist activity was isolated. The goal of this study was to purify and identify the compound responsible for that activity.

## Materials and methods

### Fungal strains

An argiudol vertic soil [total C: 3·02, pH 5·95 (1 : 2·5 V:V), N: 0·26, P extr. mg $kg^{-1}$ 24·15] of a soybean field (Salto, Buenos Aires, Argentina) with patches of suppressive and nonsuppressive soils against Sclerotinia wilt, was used for fungal isolation. *Sclerotinia sclerotiorum* strain (BAFC 225) was isolated from sclerotia of infected soybean plants in the nonsuppressive soil.

The antagonistic strain was isolated from the suppressive soil with a soil particle washing method (Parkinson 1994) and was selected according to its antagonistic behaviour in dual cultures confronting *S. sclerotiorum* (BAFC 225).

By using culture characteristics and spore morphology, the antagonistic strain was identified as *F. oxysporum* (Booth 1971; Nelson *et al.* 1983).

### Dual cultures

*Sclerotinia sclerotiorum* (BAFC 225) and *F. oxysporum* (S6) were confronted in dual cultures in Petri dishes, on two different culture media: malt extract agar (MEA) and potato dextrose agar (PDA). Two assays were conducted: *F. oxysporum* (S6) was inoculated 48 h before and at the same time as the pathogen. The first assay detected the presence of antifungal metabolites, whereas the second assessed the effectiveness of its antagonistic capacity as the antagonist strain showed a growth rate less than that of the pathogen. In both tests two 4-mm diameter plugs were inoculated 4·5 cm apart in 9 cm diameter Petri dishes. In assays in which plugs were used, they were excised from the leading edge of an actively growing colony of each fungus (Whipps 1987). Control dishes were inoculated with the pathogen strain on each of the media being assessed. A factorial design was used with four treatments per assay: *S. sclerotiorum vs F. oxysporum* (S6) in MEA, *S. sclerotiorum* alone in MEA, *S. sclerotiorum vs F. oxysporum* (S6) in PDA, *S. sclerotiorum* alone in PDA. Three replicates were included for each confrontation. All Petri dishes were kept at 25°C in darkness and the experiment was repeated twice.

The width of the inhibition zone was determined and the percentage of radial growth inhibition (%RGI) was calculated as:

$$\%\mathrm{RGI} = [(r_c - r_d)/(r_c \times 100)]$$

where $r_c$ is the control pathogen colony radius; $r_d$ is the pathogen colony radius in the dual culture (Melgarejo *et al.* 1985).

### Observations by light microscopy

After 10 days of inoculation, squares of 10 mm × 10 mm from the pathogen colonies in contact with the inhibition zone in MEA were removed. Each square was stained with cotton blue and observed by light microscopy.

### Assessment of *Fusarium oxysporum* (S6) as a mycoparasite on sclerotia

Twenty-five days old sclerotia of 2–3 mm diameter, obtained from culture on MEA, were employed in two different experiments for mycoparasitism evaluation:

*Sclerotia spore inoculation*

Forty sclerotia were submerged in a suspension of *F. oxysporum* (S6) spores in sterile water in a relation of $0{\cdot}25 \times 10^6$ sp $ml^{-1}$ $sclerotia^{-1}$ for 5 min.

Half of the sclerotia (20) were planted in a Petri dish containing sterile sand and the other half in a Petri dish containing sterile soil. For control treatments, only sterilized distilled water was used (Whipps and Budge 1990). We performed four treatments with two replicates each: (i) inoculated sclerotia in sand, (ii) control sclerotia in

  

sand, (iii) inoculated sclerotia in soil and (iv) control sclerotia in soil. Substrates were moistened up to field capacity with sterile water. All Petri dishes were kept at 25°C for 28 days in darkness. Each assay was performed twice.

From each Petri dish the following values were recorded percentage of: recovered sclerotia (%RS), colonized sclerotia (%CS), infected sclerotia by the antagonist (%IS) and viable sclerotia (%VS). Each parameter was calculated as: $\%RS = n°rs/(n°ps \times 100)$; $\%CS = n°cs/(n°ps \times 100)$; $\%IS = n°is/(n°ts \times 100)$; $\%VS = n°vs/(n°ts \times 100)$,where $n°rs$ is the number of recovered sclerotia; $n°ps$ is the number of planted sclerotia; $n°cs$ is the number of colonized sclerotia; $n°is$ is the number of infected sclerotia; $n°ts$ is the number of sclerotia randomly taken from each Petri dish and $n°vs$ is the number of viable sclerotia.

For the last two values (%IS and %VS), five sclerotia were randomly taken from each Petri dish, rinsed with water, superficially sterilized with 70% sodium hypochlorite for 3 min and finally washed with sterile distilled water. They were dried on sterile filter paper and planted on MEA with antibiotics (streptomycin 0·5% and chlorotetracyclin 0·25%).

*Effect of the* Fusarium oxysporum *colony on the sclerotia of* Sclerotinia sclerotiorum

A 4-mm diameter plug of the antagonist was centrally inoculated in a 6-cm diameter Petri dish containing MEA. After 7 days, eight sclerotia were placed equidistant from each other around 2 cm of the centre of the colony. For the control treatment, eight sclerotia were kept in sterile Petri dishes with sterile filter paper. All Petri dishes were kept at 25°C in darkness. Each procedure was performed in triplicate. Three harvesting conditions were considered: 15, 30 and 45 days after sclerotia incorporation, employing different Petri dishes for each treatment. Three sclerotia from each Petri dish were randomly sampled after 15 and 30 days, and two of them, after 45 days. Percentage of colonized sclerotia (%CS), infected sclerotia by *F. oxysporum* (S6) (%IS) and viable sclerotia (%VS) were recorded. They were calculated as in a). For %IS and %VS, harvested sclerotia were rinsed and sterilized as above. A factorial design was employed.

### Production of nonvolatile metabolites

Petri dishes containing MEA were covered with boiled sterilized cellophane (50 mm diameter). A plug (4 mm diameter) from *F. oxysporum* (S6) colony was centrally inoculated. The plates were incubated at 25°C in darkness. After 3 days, the cellophane and the colony were removed. A 4-mm plug of pathogen, excised from a 4-day-old culture, was placed at the centre of the plate. The same experiment was repeated but without inoculation of *F. oxysporum* (S6) as control. A factorial design was used with two treatments: pathogen growing with and without the presence of antagonist exudates in MEA. The plates were incubated at 25°C for 15 days in darkness, and the diameters of the pathogen colonies were measured every day (Whipps 1987). Three replicates were made for each treatment, and the experiment was repeated twice.

For fungicidal or fungistatic activity determination, the assay was repeated again, but the plates were incubated at 25°C only for 4 days after *S. sclerotiorum* plugs were placed onto the plate. Then, *S. sclerotiorum* inocula were re-inoculated onto MEA and the percentage of viable plugs was determined (Dennis and Webster 1971; Jackson *et al.* 1991). This procedure was made in triplicate and repeated twice.

### Antifungal activity during the antagonist growth

The antagonist strain *F. oxysporum* (S6) was cultured in malt extract broth (MEB) under static condition at 25°C and the efficacy of nonvolatile metabolites present in the media was studied (Fuhrmann 1994). A 4-mm plug of *F. oxysporum* (S6), taken from the growing edge of MEA culture was used to inoculate 50 ml Erlenmeyer flasks containing 20 ml of MEB. Time of incubation was determined to relate antifungal activity of exudates and antagonistic strain growth, by measuring mycelium dry weight at 1, 3, 5, 7 and 10 days after inoculation. Individual 20 ml cultures were vacuum-filtered (Whatman No. 1 filter paper) and the mycelium was oven-dried at 70°C for 48 h. Each sample time was made in duplicate. Petri dishes were prepared with the broth (filtered by Millipore filter 0·2 $\mu$m) incorporated into fresh MEA (10% concentration v/v). A factorial design was employed with two treatments: colony growing with or without *F. oxysporum* (S6) exudates corresponding to each harvesting time. The experiment was performed in duplicate. Control dishes were prepared by using filtered culture medium (MEB at 10% concentration in MEA). A 4-mm pathogen plug was centrally inoculated in each Petri dish and incubated at 25°C.

After 4 days of incubation, the percentage of pathogen colony growth inhibition (%GI) was calculated as: $\%GI = [(D_1 - D_2) \times 100]/D_1$, where $D_1$ is the diameter of control pathogen colony and $D_2$ is the diameter of the pathogen colony when the antagonist exudates were added. Twenty days after the inoculation, the number of sclerotia and dry weight per sclerotia were determined for the pathogen colony growing in both treatments. Also, morphological changes in the colony were described.

### Fermentation

A 5-mm plug of the *F. oxysporum* colony on MEA was used to inoculate 250 ml Erlenmeyer flasks containing 100 ml of MEB. After a week, this culture was employed to inoculate a 4 l Erlenmeyer flask containing 1 l of MEB. Incubation was carried out at 25°C for 21 days under static conditions.

### Extraction and isolation of the active compound

The culture (1 l) was filtered and the filtrate partitioned with EtOAc. The mycelia was washed with distilled water and extracted with EtOH and EtOAc. Both organic extract media and mycelia were evaporated to dryness.

The mycelia extract was vacuum-chromatographed on reversed phase silica gel by using $H_2O$ and mixtures of $H_2O$-MeOH of increasing polarity. The active fraction was further chromatographed on HPLC [Column: YMC C 18, 5 $\mu$m, 22·5 × 2·5 cm; Eluant: MeOH-$H_2O$ 85 : 15 (v/v); RI and UV (215 nm) detection], yielding pure active cyclosporine A (60 mg). The medium extract was treated in the same way to yield cyclosporine A (5 mg).

*Cyclosporine A*. $[\alpha_D] = -192$ (MeOH, $c = 0{\cdot}0113$; −189 (Traber *et al.* 1987)) FAB $^+$ MS (matrix: 3-NBA): *m/z* 1202 $(M + H)^+$ . $^1$ H NMR ($CDCl_3$): 1-*MeBmt*: $\delta$ 5·46 (H-2), 5·34 (H-6, 7), 3·80 (H-3), 3·50 ($NCH_3$), 2·39 and 1·67 (H-5), 1·63 (H-4, 8), 0·72 (H-9); 2-*Abu*: $\delta$ 7·93 (N*H*), 5·03 (H-2), 1·69 (H-3), 0·86 (H-4); 3-*Sar*: $\delta$ 4·73 (H-2), 3·39 ($NCH_3$), 3·20 (H-2); 4-*MeLeu*: $\delta$ 5·33 (H-2), 3·10 ($NCH_3$), 2·00 and 1·60 (H-3), 1·43 (H-4), 0·94 (H-5), 0·88 (H-6); 5-*Val*: $\delta$ 7·46 (N*H*), 4·66 (H-2), 2·41 (H-3), 1·06 (H-4), 0·89 (H-5); 6-*MeLeu*: $\delta$ 4·99 (H-2), 3·25 ($NCH_3$), 2·05 (H-3), 1·75 (H-4), 1·40 (H-3), 0·93 (H-5); 0·84 (H-6); 7-*Ala*: $\delta$ 7·64 (N*H*), 4·51 (H-2), 1·35 (H-3); 8-*Ala*: $\delta$ 7·16 (N*H*), 4·83 (H-2), 1·26 (H-3); 9-*MeLeu*: $\delta$ 5·70 (H-2), 3·11 ($NCH_3$), 2·11 (H-3), 1·32 (H-4), 1·27 (H-3), 0·96 (H-5), 0·88 (H-6); 10-*MeLeu*: $\delta$ 5·07 (H-2), 2·70 ($NCH_3$), 2·09 (H-3), 1·49 (H-4), 1·25 (H-3), 1·02 (H-5), 1·01 (H-6); 11-*MeVal*: $\delta$ 5·15 (H-2), 2·71($NCH_3$), 2·15 (H-3), 1·00 (H-4), 0·85 (H-5).$^{13}$ C NMR ($CDCl_3$): *MeBmt*: $\delta$ 129·6 and 126·2 (C-6,7), 74·7 (C-3), 58·8 (C-2), 36·0 (C-4), 35·6 (C-5), 17·9 (C-8), 16·7 (C-9); *Abu*: $\delta$ 48·8 (C-2), 24·9 (C-3), 9·8 (C-4); *Sar*: $\delta$ 50·3 (C-2); *MeLeu*: $\delta$ 55·5 (C-2), 36·0 (C-3), 24·8 (C-4), 23·4 (C-5), 21·1 (C-6); *MeVal*: $\delta$ 48·3 (C-2), 29·0 (C-3), 20·2 (C-5), 18·7 (C-4); *Val*: $\delta$ 55·4 (C-2), 31·1 (C-3), 19·8 (C-4), 18·4 (C-5); *MeLeu*: $\delta$ 55·3 (C-2), 37·4 (C-3), 25·3 (C-4), 23·8 (C-5), 21·9 (C-6); *Ala*: $\delta$ 48·6 (C-2), 15·9 (C-3); *Ala*: $\delta$ 45·1 (C-2), 18·1 (C-3); *MeLeu*: $\delta$ 48·3 (C-2), 39·0 (C-3), 24·6 (C-4), 23·6 (C-5), 21·8 (C-6); *MeLeu*: $\delta$ 57·5 (C-2), 40·7 (C-3), 24·5 (C-4), 23·8 (C-5), 23·3 (C-6); *C*O: 173·7, 173·6, 173·5, 173·4, 171·6, 171·1 (×2), 170·4, 170·3, 170·1, 170·0.

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker AM-500 instrument (Bruker, Billerica, MA, USA) at 500·1 MHz for $^1$H and at 125·13 MHz for $^{13}$C NMR. Optical rotations were measured on a Perkin Elmer 343 polarimeter (Perkin Elmer, Wellesley, MA, USA). FAB$^+$/MS was recorded at the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry. Department of Chemistry, St. Louis, MO.

### Antifungal activity of the fractions and MIC determination

The antifungal activity for bioguided fractionation was determined by the agar diffusion method by using 100 $\mu$g of sample per disc against *S. sclerotiorum* (Strobel *et al.* 1999). After isolation and purification of the metabolite, the minimum inhibitory concentration (MIC) was determined by using the same method. Different concentrations of the metabolite were employed: 0·05, 0·10, 0·20, 0·50, 1·00, 5·00, 10·00 and 20·00 $\mu$g of sample per disc. MIC was determined as the minimum assayed concentration at which a zone of inhibition was obtained.

### Cyclosporine A quantification

*Fusarium oxysporum* (S6) strain was cultured as above; a plug was incorporated in a 50-ml flask with 20 ml MEB. The flasks were incubated in static conditions at 25°C in the darkness. A daily harvest of two flasks was made. Every sample was filtered, extracted and pH and dry weight mycelium were measured. The medium was subjected to an SPE cartridge (C18, 6 ml), washed with $H_2O$ (10 ml) and eluted with MeOH (10 ml). The mycelium was extracted with EtOH (20 ml) and treated with ultrasound for 2 min. After 24 h it was vacuum-filtered. The active organic fractions (fraction eluted with MeOH from medium –f1- and the mycelium extract –f2-) were dried and weighed. Then, 200 $\mu$l of MeOH : DMSO 1 : 1 and 1 : 3 was added to f1 and f2, respectively. F2 was filtered through an inorganic membrane filter (0·2 $\mu$m, Anotop, Whatman). Both medium and mycelium samples were analysed by HPLC to determine cyclosporine A concentration. Concentrations were measured by reversed phase HPLC (Pinnacle II, C8, 5 $\mu$m, 150 × 4·6 mm, Restek) in linear gradient (1 ml min$^{-1}$ flow rate) with MeOH : $H_2O$ (30 : 70) to MeOH 100% in 15 min monitoring the absorbance at 220 nm.

A Gilson chromatograph with Rheodyne injector and diode array detector was employed.

### Biocontrol capacity in greenhouse experiment

A greenhouse experiment was conducted by using Asgrow 5409 soybean seeds. The *F. oxysporum* (S6) inoculum was

  

added to the steam-pasteurized soil as a mass of mycelium and spores growing on boiled rice at a concentration of $1{\cdot}3 \times 10^6$ CFU $g^{-1}$ (colony-forming units per gram of soil: CFU $g^{-1}$) . Pregerminated seeds were planted in 250-ml pots containing 200 ml of steam-pasteurized soil inoculated with the antagonist strain. After 3 days, the plants and the inoculated soil were transferred to 600-ml pots containing 300 ml of inoculated soil with *S. sclerotiorum* inoculum (concentration: 15%, w/v), containing pathogen mycelia and sclerotia. This inoculum was produced in sterile polythene bags containing rice : bran : water (20 : 20 : 100; V : V : V) as substrate inoculated with 5-mm pathogen plugs (six per 350 g of substrate) and incubated for 20 days at 24–28°C in the darkness. A completely randomized factorial design was used. We performed four treatments with four replicates each: pathogen only (treatment S), antagonist only (treatment F), pathogen + antagonist (treatment S + F) and control (C) – with neither antagonist nor pathogen. The control plants received the same substrates at the same proportion, but without the inoculum. The plants grew in a greenhouse for a month, until they fruited. The experiment was conducted twice.

Percentage of surviving plants, shoot length, dry weight of roots and shoots were recorded.

### Statistical analysis

Analysis of variance was performed at the significance level of $P < 0{\cdot}05$. When appropriate, means were separated by using Tukey's test ($P < 0{\cdot}05$).

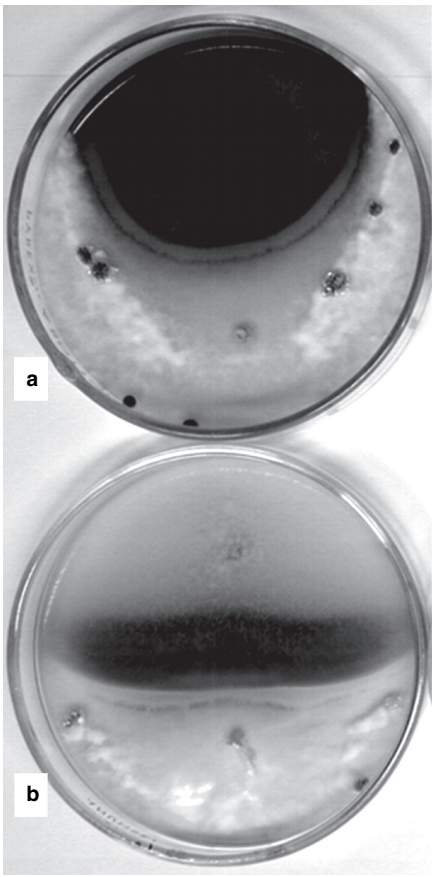


**Figure 1** Dual cultures where *F. oxysporum* (S6) was inoculated 48 h before *S. sclerotiorum* (after 10 days): (a) on malt extract agar (MEA); (b) on potato dextrose agar (PDA).

## Results

### Dual cultures

The experiments on antagonist activity showed that *F. oxysporum* (S6) significantly inhibited pathogen growth on both media assessed (MEA and PDA) with differences on inhibition halo width whether it was planted simultaneously or 48 h earlier. An inhibition zone was obtained on both culture media (Table 1), but in PDA, the antagonist grew to the edge of the pathogen colony, making the inhibition zone significantly smaller than on MEA (Fig. 1). The greatest inhibition percentages were found on PDA (Table 1) where there was less necrosis of the pathogen colony compared to MEA (Fig. 1). The %RGI and the inhibition zone remained stable during the experiments. The colour of the *F. oxysporum* (S6) colony differed in the two media. On PDA it was pink-white (5YR 8/1- Munsell Color Co. 1954), and violet (5YR 6/1 Munsell Color Co. 1954) when the colony confronted the pathogen. On MEA it was pink (10R 5/4 Munsell Color Co. 1954) and a red pigment was released into the med-

**Table 1** Percentage of radial growth inhibition (%RGI), percentage of sclerotia formation inhibition (%SFI), width of inhibition zone (Iz) for *S. sclerotiorum* in dual cultures with *F. oxysporum* (S6) on MEA and PDA in simultaneous inoculation and deferred inoculation. Values represent the means for each treatment with the standard deviation

| | MEA | | | PDA | | |
|---|---|---|---|---|---|---|
| | %RGI | %SFI | Iz (mm) | %RGI | %SFI | Iz (mm) |
| Simultaneous inoculation | 59·44 ± 6·12 | 40·75 ± 26·68 | 2·00 ± 0·80 | 78·33 ± 2·79 | 51·5 ± 8·81 | 0·30 ± 0·50 |
| Deferred inoculation | 67·88 ± 1·11 | 40·76 ± 7·90 | 3·50 ± 0·60 | 77·22 ± 4·21 | 42·68 ± 16·89 | 0·50 ± 0·60 |

ium. This pigment was of a more intense colour (10R 4/6- Munsell Color Co. 1954) when the antagonist colony confronted *S. sclerotiorum* and appeared several days before that in the control colony. This difference in behaviour between the control colony and the confronted colony was not observed on PDA.

### Observations by light microscopy

The mycelium of *S. sclerotiorum* in contact with the inhibition zone showed marked necrosis on MEA (Fig. 1a). Mycelium alterations included increased branching, reduced length of branches, granulation, retraction of the plasmalemma and collapse of cytoplasm (Fig. 2). No differences were found in either of the inoculation times.

### Production of nonvolatile metabolites

The cellophane test showed that the antagonistic strain produced diffusible metabolites with antifungal activity against *S. sclerotiorum*. Also, diffusion of the red pigment from the *F. oxysporum* (S6) colony was observed. The *S. sclerotiorum* inoculum showed marked melanization and mycelium did not develop throughout the 15 days of the assay. After 6 days, the pathogen strain planted in fresh MEA began to grow.

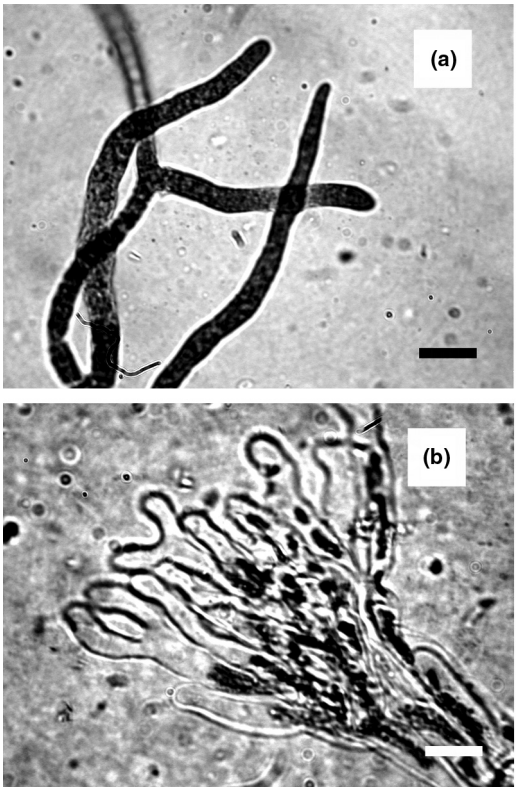


**Figure 2** Alterations of the *S. sclerotiorum* mycelium: (a) edge of the colony in the control; (b) mycelium in contact with *F. oxysporum* (S6) inhibition zone on MEA, collapsed cytoplasm and branched hyphae. Scale bar: 10 $\mu$m.

### Evaluation of the capacity of *Fusarium oxysporum* as a mycoparasite on *Sclerotinia sclerotiorum* sclerotia

When spores were used for inoculation, all the sclerotia were recovered from both sand and soil. At the end of the assay, differences were found between substrate. The *F. oxysporum* (S6) strain was able to colonize the sclerotia superficially only in soil and the antagonist was re-isolated only from 6·67% of them. Also, there was a slight reduction of the sclerotia viability (Table 2).

When the sclerotia were planted in the centre of the *F. oxysporum* colony, there was a nonsignificant increase of sclerotia infection, but the viability decreased (Table 3). In fact, percentages of viable sclerotia were significantly lower after 30 and 45 days.

### Relationship between growth and antagonistic activity

A marked reduction in the growth rate of the pathogen was obtained when the *F. oxysporum* (S6) culture filtrate was added on the media (Fig. 3a). The percentage of growth inhibition (%GI) of the pathogen colony was significantly larger when the filtrate from a 7-day-old culture (d7) of *F. oxysporum* (S6) was used. Figure 4 shows the growth inhibition percentages obtained along the time (GI%) with *F. oxysporum* (S6) exudates culture. Also, there was a significant reduction in the number of sclerotia produced and their average dry weight was significantly larger when filtrates from d7 and d10 were added

**Table 2** Mycoparasitism of *S. sclerotiorum* sclerotia by *F. oxysporum* (S6) inoculated as spore suspension and incubated in sand or soil as substrate and evaluated as: percentage of recuperated sclerotia (%RS), percentage of colonized sclerotia (%CS), percentage of infected sclerotia (%IS) and percentage of viable sclerotia (%VS). In each substrate two treatments were employed: *F. oxysporum* (S6) spore inoculated sclerotia and control sclerotia (without *F. oxysporum* inoculation). There were no significant differences between treatments (ANOVA $P < 0{\cdot}05$)

| | Sand | | Soil | |
|---|---|---|---|---|
| | Inoculation with S6 spores | Control | Inoculation with S6 spores | Control |
| %RS | 100·00 | 100·00 | 100·00 | 100·00 |
| %CS | 0·00 | 0·00 | 26·67 | 0·00 |
| %IS | 0·00 | 0·00 | 6·67 | 0·00 |
| %VS | 100·00 | 100·00 | 93·33 | 100·00 |


 

**Table 3** Mycoparasitism in sclerotia incorporated into *F. oxysporum* (S6) colony (treatment FS6) and in sclerotia control over time evaluated as: percentage of colonized sclerotia (%CS), percentage of infected sclerotia (%IS) and percentage of viable sclerotia (%VS). In each substrate two treatments were employed: *F. oxysporum* (S6) spore inoculated sclerotia and control sclerotia (without *F. oxysporum* inoculation). Different letters indicate significant differences between treatments (ANOVA Tukey test $P < 0{\cdot}05$)

| | Day 15 | | Day 30 | | Day 45 | |
|---|---|---|---|---|---|---|
| | FS6 | Control | FS6 | Control | FS6 | Control |
| %CS | 83·33 b | 0·00 a | 100·00 b | 0·00 a | 100·00 b | 0·00 a |
| %IS | 11·11 a | 0·00 a | 11·11 a | 0 a | 16·67 a | 0·00 a |
| %VS | 88·90 a | 100·00 a | 44·44 b | 100 a | 50·00 b | 100·00 a |

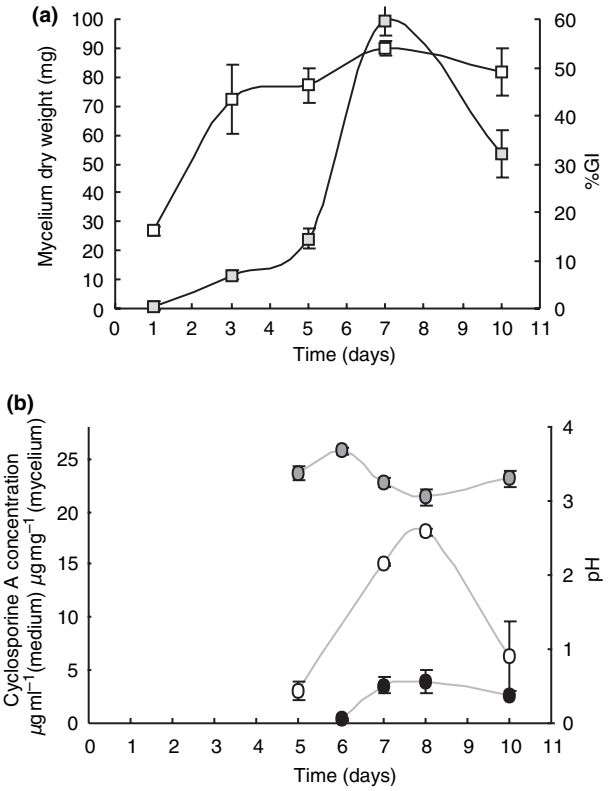


**Figure 3** Antifungal activity and cyclosporine A production: (a) *F. oxysporum* (S6) growth curve in malt extract medium broth (MEB) measured as dry weight of mycelium (□) and antifungal activity presented by the medium as percentage of growth inhibition (%GI) of the *S. sclerotiorum* colony on MEA when that medium was added at a concentration of 10% (v/v) (■). (b) Cyclosporin A quantification, analysed by HPLC and measured as micrograms ($\mu$g) of compound per millilitre of medium (●) and micrograms of compound per milligram of dry mycelium (○) over time. Also, pH was determined at each harvesting day (◍). Bars indicate standard deviations of the mean.

to the growing media of *S. sclerotiorum* (Fig. 5). Pathogen colonies showed noticeable alterations such as reduction of aerial mycelium with irregular borders and slight melanization. Also, white structures such as immature sclerotia were observed.

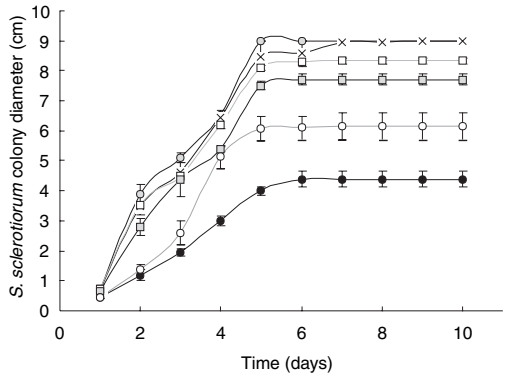


**Figure 4** Growth curve of the pathogen *S. sclerotiorum* on solid medium measured as colony diameter over time with the addition of 10% (v/v) of the filtrate obtained *F. oxysporum* (S6) culture at different days: d1 (1 day old culture) (×); d3 (3 days old culture) (□); d5 (5 days old culture) (■); d7 (7 days old culture) (●); d10 (10 days old culture) (○); control (◍). Bars indicate standard deviations of the mean.

### Extraction and isolation of the antifungal compound

The partition of the crude extracts of mycelia and medium of *F. oxysporum* (S6), guided by antifungal activity against *S. sclerotiorum*, yielded a pure compound (MIC 0·1 $\mu$g disc$^{-1}$).

The isolated compound exhibited four signals as doublets between 7·0 and 8·0 ppm, eleven multiplets between 4·4 and 5·8 ppm, seven methyl groups attached to nitrogen between 2·7 and 3·5 ppm, besides other signals in the $^{1}$H NMR ($^{1}$H nuclear magnetic resonance) spectrum. Both this fact and the presence of 11 carbonyl groups in the $^{13}$C NMR spectrum suggested that the pure compound was a peptide of 11 amino acids, seven of them N-methylated, as shown by $^{1}$H NMR. The two-dimensional (2D) NMR experiments, HETCOR (Heteronuclear Chemical Shift Correlation) and COSY H,H (homonuclear correlation spectroscopy) allowed to establish the connectivities between protons and carbons directly attached, and between vicinal protons, respectively. A 2D-*J* experiment was employed to discriminate

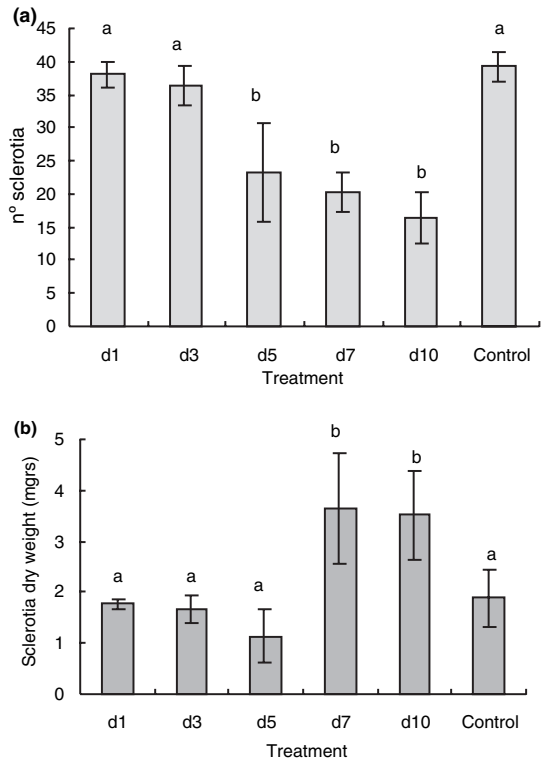


**Figure 5** (a) Number per plate and (b) dry weight of sclerotia formed in the *S. sclerotiorum* colony on malt extract agar. *F. oxysporum* (S6) was grown in malt extract medium broth (MEB). After 1, 3, 5, 7 and 10 days, *F. oxysporum* media was filtered and added at a concentration of 10% to malt extract agar (MEA) (v/v). Different letters indicate significant differences between treatments (anova Tukey test $P < 0{\cdot}05$). Bars indicate standard deviations of the mean.

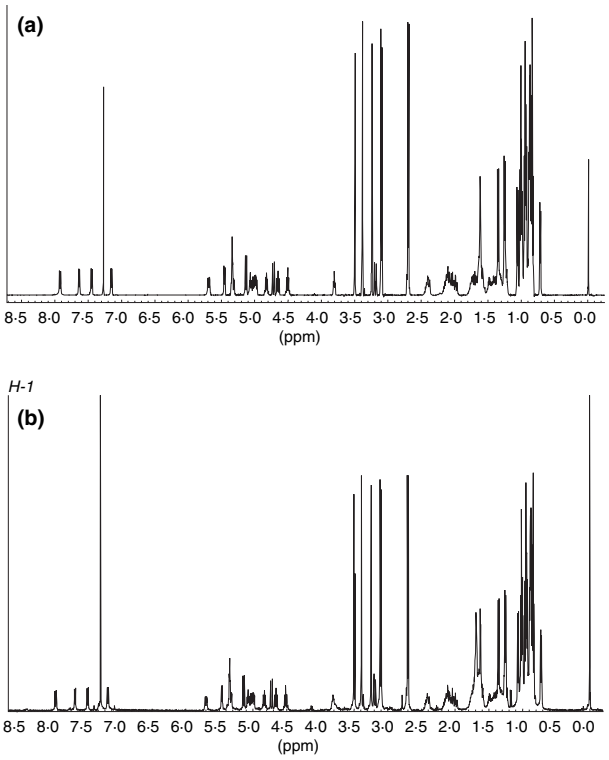


**Figure 6** $^{1}$H NMR spectra of cyclosporine A from *F. oxysporum* (S6) (a) and commercial sample (b).

between overlapped proton signals. All these data determined the presence of four MeLeu, two Ala, and one MeBmt, Abu, Sar, Val and MeVal. As these amino acids are present in cyclosporine A (CyA), this compound was isolated from a commercial sample of Sandimmun® (Novartis) in order to compare $^{1}$H and $^{13}$C NMR spectra. Both the spectra (Fig. 6) and the optical rotation were identical for both compounds. For these reasons, the isolated compound was unambiguously identified as cyclosporine A (Fig. 7).

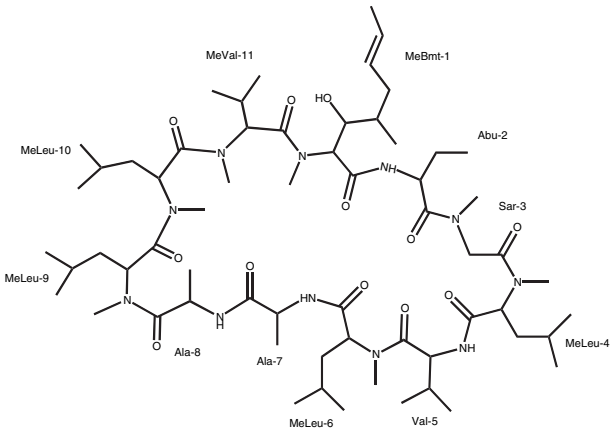


**Figure 7** Cyclosporine A.

## Cyclosporine A quantification

The production of cyclosporine A initiated on day 5 and the amount of cyclosporine A reached a maximum on days 7 and 8, the same as the antifungal activity (Fig. 3b). The maximum production found in medium and in mycelium was 3·88 $\mu$g ml$^{-1}$ and 18·1 $\mu$g mg$^{-1}$, respectively. Associated with that largest metabolite production, there was a medium pH decrease.

## Assessment of the biocontrol capacity of the antagonist strain (greenhouse experiments)

The greenhouse experiments showed a reduction in the percentage of diseased soybean plants when they were pretreated with the antagonistic strain (Fig. 8). The percentage of surviving plants was significantly lower for plants inoculated with the pathogen (treatment S) than


 

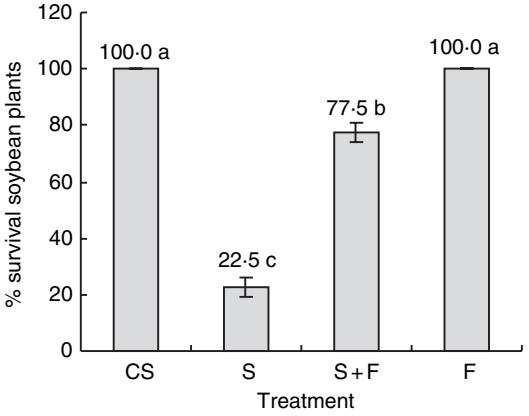


**Figure 8** Biocontrol experiments with *S. sclerotiorum* (pathogen) and *F. oxysporum* (S6) (biocontrol agent) in soybean plant under greenhouse conditions. Percentage of surviving plants for the four treatments: plants with *F. oxysporum* (S6) inoculation (F); with *S. sclerotiorum* (S); plants with *F. oxysporum* (S6) and *S. sclerotiorum* inoculation (F + S) and control [without *F. oxysporum* (S6) or *S. sclerotiorum* inoculation]. Different letters indicate significant differences between treatments (anova Tukey test $P < 0{\cdot}05$). Bars indicate standard deviations.

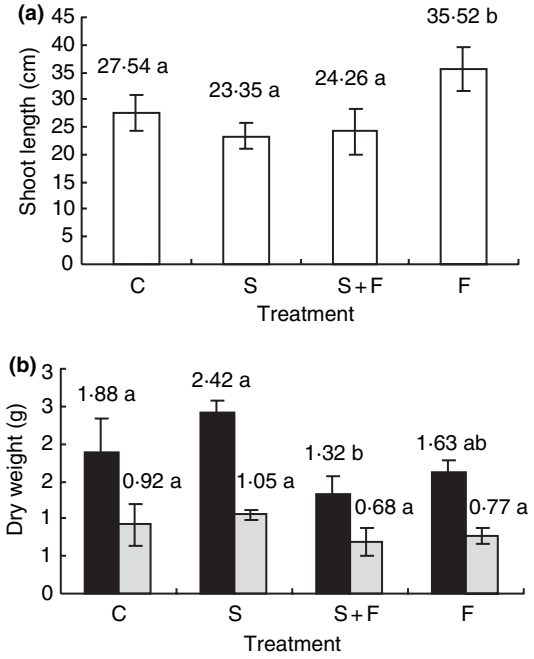


**Figure 9** Growth parameters in the greenhouse experiments: length □ (a) and dry weight of shoots ■ and dry weight of roots ▩ (b) of surviving plants in the biocontrol experiments with *F. oxysporum* (S6) confronting *S. sclerotiorum* in four treatments. Plants inoculated with *F. oxysporum* (S6) (F); *S. sclerotiorum* (S); *F. oxysporum* (S6) and *S. sclerotiorum* (S + F) and control [without *F. oxysporum* (S6) or *S. sclerotiorum* inoculation]. Different letters (anova Tukey test $P < 0{\cdot}05$) indicate significant differences between treatments. Bars indicate standard deviations.

for plants in which *S. sclerotiorum* was inoculated with *F. oxysporum* (S6) (treatment S + F).

Under the experimental conditions, the presence of *F. oxysporum* (S6) alone (treatment F) did not affect the percentage of surviving plants.

At the end of the experiment, there were significant differences in shoot length and in dry weight of shoots of surviving plants. In plants under treatment F, shoot length was significantly larger (Tukey unequal *N*; $P < 0{\cdot}05$). Shoot dry weight was significantly lower in plants from treatment S + F than in control plants (treatment C) (Tukey unequal *N*; $P < 0{\cdot}05$). Shoot dry weight in plants from treatment F was intermediate between values for treatments C and S + F and did not differ significantly from them (Tukey unequal *N*; $P < 0{\cdot}05$). There was no significant difference in the dry weight of roots in any case (Fig. 9).

## Discussion

*Fusarium oxysporum* is well known for its ability to grow and sporulate profusely in soil, when large amount of nutrients are available, overcoming soil fungistasis (Papavizas 1985). It has the ability to colonize the rhizosphere and roots of different plant species (Fravel *et al.* 2003) and it is present with high frequency in suppressive soybean soils against *S. sclerotiorum* (Rodríguez 2004).

Our *in vitro* assays showed that the *F. oxysporum* (S6) strain has a marked antagonistic capacity against the pathogen *S. sclerotiorum*, producing significant reduction in the growth in all the experimental conditions assayed. Potentially the *in vitro* tests, such as dual cultures, could indicate the potential of some organisms to produce chemicals or to act as mycoparasites, and could be used to delineate the range of variables from which both optimal use of the antagonist and control of certain pathogens can be obtained (Whipps 1987). Antagonist strains of *Trichoderma harzianum* and *Coniothyrium minitans* have shown antagonistic effect in *in vitro* assays (Whipps 1987) and different isolates of these species have been evaluated as potential biocontrol agents of *S. sclerotiorum* and other species in this genus (Zhou and Boland 1998). *F. oxysporum* (S6) showed inhibition of mycelium growth and sclerotia production, so it may therefore be considered as a potential biocontrol agent.

Hyphal alterations have been described in dual cultures of *S. sclerotiorum* with different *Fusarium* species (Zazzerini and Tosi 1985), but the microscopic alterations were less evident than the ones we found in our experiments. The melanization present in the pathogen mycelium in dual cultures involves the deposition of polymers and phenolic and/or indolic compounds in the tissues

(Nosanchuk and Casadevall 2003) protecting cells against physical and biological stress, and increasing hyphal resistance to cell wall-degrading enzymes (Butler *et al.* 2001). In our experiments, the melanization produced in the *S. sclerotiorum* colonies might be because of the response of the pathogen against a toxic metabolite produced by *F. oxysporum* (S6). This response may be more efficient in MEA than in PDA, where less melanization and greater %RGI was obtained, suggesting that it could be related to the less efficient protection of the pathogenic colony. This fact would indicate that the nutritional conditions could be involved in the protection response of the pathogen.

Both mycoparasitic capacity and superficial colonization with symptoms of softening in some strains of different *Fusarium* species have been reported (Zazzerini and Tosi 1985). In this sense, our results showed differences with those isolates. *Fusarium oxysporum* (S6) colonized the sclerotia superficially, but did not produce the softening mentioned above. In both mycoparasitim evaluation assays, *F. oxysporum* (S6) reduced the viability of this resistant structure in the soil, so it could be used to reduce this important inoculum source.

The cellophane test provided evidence of the presence of a nonvolatile metabolite that can diffuse into the medium and that completely inhibits the pathogen mycelia growth. The dilution assay using antagonist exudates provides an adequate tool to confirm the diffusible nature of nonvolatile metabolites (Hadacek and Greger 2000) and antifungal activity (Hostettmann 1991). The dilution assay was also used to demonstrate the possible role of antibiosis in biocontrol (Fravel 1988), which is a well-documented phenomenon in soil-borne antagonist fungi (Whipps 2001). The dilution experiments showed that the 10% dilution (v/v) produced a significant reduction of pathogen growth and number of sclerotia. The metabolite responsible for the antifungal activity was identified as cyclosporine A, which is well known as an immunosuppressor (Carlile *et al.* 2001).

This is the first time that the antifungal activity of cyclosporine A was described against the phytopathogenic fungus *S. sclerotiorum*. However, antifungal activity of cyclosporine A against other fungal strains such as some species of the genera *Aspergillus, Curvularia, Trichophyton, Rhodotorula* and *Neurospora* (Dreyfuss *et al.* 1976, Cruz *et al.* 2000) has been linked to the inhibition of chitin synthesis (Dreyfuss *et al.* 1976). The antimicrobial activity of cyclosporine A has been related to the intracellular receptor cyclophilin A (Foor *et al.* 1992). The complex cyclophilin A–cyclosporine A might work as a potent inhibitor of different proteins, important in calcineurin metabolism (Breuder *et al.* 1994). In fact, the cyclosporine A–cyclophilin A complex has been linked with calcineurin inactivation, involved in the infection of *Magnaporthe grisea*. Calcineurin is required for appressorium morphogenesis and the regulation hyphal growth and development in this phytopathogenic fungus (Viaud *et al.* 2002). The cyclosporine A antifungal activity spectra have been compared with those of polioxins (Dreyfuss *et al.* 1976), which are employed as fungicides for agriculture (Monaghan and Tkacz 1990).

The purification and identification of the metabolite could improve our understanding on the mechanism involved in this system. The production of cyclosporine A was quantified both in medium and mycelium, showing a correlation between metabolite concentration and antifungal activity. The greatest concentration was in the mycelium. This method could allow us to investigate the effect of different growth conditions to reach greater antifungal activity from the strain, through metabolite quantification.

The significant reduction in the number of sclerotia and their viability would greatly reduce the pathogen inoculum source, thus making *F. oxysporum* (S6) and cyclosporine A potential biocontrol sources.

The soybean plants inoculated only with the antagonist strain showed a significant increase of shoot length and no symptoms of disease. For this reason, this strain may be considered as nonpathogenic. Considering the reduction in the number of diseased plants when the antagonist strain was inoculated together with the pathogen, we concluded that *F. oxysporum* (S6) strain may be a suitable biocontrol agent.

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